Folia Zool. – 53(4): 433–436 (2004)

A contribution to the genetic characterisation of some species of the genus *Gobio* (Cyprinidae)

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Received 25 November 2003; Accepted 15 December 2004

A b s t r a c t . RAPD-PCR method is now widely employed in genetic research and is a powerful asset when trying to identify species. In the present work, RAPD genetic markers were obtained for distinguishing between three species of the genus *Gobio* that show substantial morphological resemblance. Different profiles were observed: six species-specific markers were detected for *G. gobio*, four for *G. kesslerii* and nine for *G. uranoscopus*. These markers were reproducible, dependable and very helpful in differentiating between these species.

Key words: *Gobio*, RAPD, diagnostic marker, conservation

Introduction

The development of molecular technologies has provided researchers in many different fields with powerful new tools. Since 1990, many laboratories have used DNA fingerprinting technology; in particular, the use of RAPD-PCR (random amplified polymorphic DNA based on the polymerase chain reaction; W i l l i a m s et al. 1990, W e l s h & M c C l e l l a n d 1990) has become very common. This technique involves the amplification of random segments of genomic DNA using a single primer of arbitrary nucleotide sequence. The polymorphisms detected in the nucleotide sequence can then be used as genetic markers. As in other DNA methodologies, the RAPD technique has some drawbacks, in this case the dominance of markers and reproducibility. Nonetheless, it has many advantages over other molecular methods: it is faster and less labour intensive than other DNA procedures, only small amounts of DNA are required, and no information on the genome under investigation is necessary. Nowadays, RAPD markers are invaluable tools in species differentiation and identification.

The genus *Gobio*, which belongs to the group of so-called 'small fishes', has received relatively little attention because of its low economic importance. At present, nine species are known in Europe (K o t t e l a t 1997, N a s e k a & B o g u t s k a y a 1998); national and European laws protect some of them because their existence is endangered. These species bear a strong morphological resemblance to one another, and this has led to the misidentification of Central European gudgeons for many years (F r e y h o f et al. 2000). Consequently, accurate information on their distribution is still scarce. Just one study on the genetic variability of *G. gobio* has been reported (S c h r e i b e r 2002).

Against this background, the Department of Ichthyology of The Institute of Vertebrate Biology of Brno (Czech Republic) launched a program to obtain more data on the genus *Gobio*, especially on its present biogeography and the status of populations. In collaboration

with the Department of Genetics of the University Complutense of Madrid (Spain), RAPD techniques were used to generate markers for identifying the species of this genus. This work will provide important information regarding the conservation of *Gobio* species.

Material and Methods

Nineteen specimens belonging to three different *Gobio* species from six sampling sites were analysed. Six individuals were morphologically identified as common gudgeon, *Gobio gobio* (Linnaeus, 1758), six as sand gudgeon, *Gobio kesslerii* Dybowski, 1862 and seven as stone gudgeon, *Gobio uranoscopus* (Agassiz, 1828). Table 1 shows the sampling sites and the number of individuals sampled from each species.

Table 1. Species of gudgeon analysed, sample sizes (N) and sites-river.

Species	N	Sampling River
Gobio gobio	2	Bečva
	3	Rožnovská Bečva
	1	Váh
Gobio kesslerii	6	Bečva
Gobio uranoscopus	3	Ublianka
	4	Tisa-Transcarpathian

The study of endangered species calls for the non-invasive collection of tissue samples (such as scales or fin clipping) to avoid the sacrifice of the fish. Therefore, total genomic DNA was isolated from fin tissue (preserved in ethanol after collection) according to the procedures of Hillis & Moritz (1990) with slight modifications. About 40 mg of fin were incubated in 500 µl of cell lysis buffer (100mM NaCl, 50mM Tris-HCl pH 8.0, 10 mM EDTA, plus 15 µl SDS 20% and 15 µl proteinase K 20mg/ml) for 40 min at 50 ºC. Total DNA was purified with two phenol-chloroform-isoamyl alcohol (25:24:1) extractions and with one chloroform extraction. The DNA was precipitated in isopropyl alcohol, pelleted by centrifugation, washed with 70% ethanol and resuspended in 150 µl of TE buffer (10mM Tris, 1mM EDTA pH 8.0). The DNA concentration was diluted to 5 ng/µl solutions for amplification reactions.

RAPD profiles were obtained using four decamer primers from Operon Technologies (C-05, C-08, C-11 and A-13). Amplifications were performed according to the protocol of Williams et al. (1990) (with some modifications) in a 12.5 µl reaction volume containing 12.5 ng of fish DNA, 2.5 pmoles of primer, 100µM of each dNTP, 4mM $MgCl_2$, 1.25 µl Stoffel buffer 10x and 0.6 Units of Stoffel Fragment DNA polymerase (Applied Biosystems).

Amplifications were performed in an M.J. Research PT-100 thermal cycler. The reaction mixture was preheated at 94º for 5 min followed by 45 cycles of amplification (1 min at 94 ºC, 1 min at 36 ºC and 6 min at 72 ºC). A final step was made at 72 ºC for 6 min. Each amplification reaction was performed at least twice: the results were consistently reproducible.

The DNA fragments amplified were resolved electrophoretically on 2% agarose gels containing 1µg/µl ethidium bromide, using a TAE buffer system (40mM Tris, 20 Mm acetic acid and 1 mM EDTA). The gels were visualised with UV light. The size of the amplification products was inferred by comparison with a 100 bp ladder standard using Multi-Analyst

Software BioRad–1997. Fragments were classified by their generating primer and size, e.g., the band C-05/705 refers to a band 705 bp long amplified by primer C-05.

Results and Discussion

The amplification of genomic DNA from the 19 gudgeon specimens with the four primers clearly showed the three *Gobio* species to have different RAPD profiles (Fig. 1). The bands obtained ranged in size from 350 to 1500 bp. For some authors, the main shortcoming of this technique is its sensitivity to changes in reaction conditions, but the present results were routinely repeatable, even though some samples had been preserved in alcohol for more than two years.

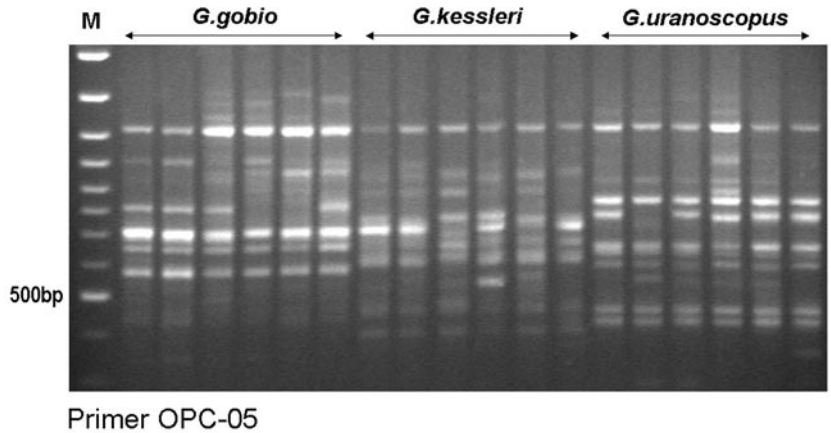

Fig. 1. RAPD profiles obtained with primer C-05 for the three species of gudgeons analysed. The first lane contains a 100bp ladder molecular weight marker. Lanes 2–7 correspond to different samples of *G. gobio*, lanes 8–13 to *G. kesslerii* and lanes 14–19 to *G. uranoscopus*.

A total of 19 DNA diagnostic markers (i.e., bands present in all individuals of one species but absent in all others) were found. Table 2 shows the specific markers for each *Gobio* species: six for *G. gobio*, 4 for *G. kessleri* and 9 for *G. uranoscopus* which allow the genetic characterisation and discrimination of each. Despite the small number of samples analysed, as the number of specific markers increases, the capacity to identify the different species also increases (C a m p t o n 1987). Therefore, this RAPD analysis successfully identified 19 DNA markers (between all the primers tested) that accurately distinguish between these three species of gudgeon. The technique is robust, reliable, reproducible and relatively easy to use with these species.

Although knowledge on how to distinguish between *Gobio* species has improved in recent years (B a n a r e s c u 1999), their identification is still not easy because of

Table 2. Size (bp) of the diagnostic bands for each species and primer.

Primer	*G. gobio*	*G. kesslerii*	*G. uranoscopus*
C-05	705, 565	469, 413	851, 644, 603, 463, 440
C-08	509	676	690
C-11	589, 471		1155, 638
A-13	475	490	565

extraordinary phenotypic diversity. However, the combination of morphological and molecular markers will doubtlessly allow fast and accurate discrimination between them.

RAPD analysis has several advantages over other molecular techniques. No prior knowledge about particular sequences or genes is required - meaning that a genetic analysis of a species can be performed without a classic genetic database being available (H a d r y s et al. 1992, H a r r y et al. 1998) - and, in contrast to other types of analysis, only small amounts of biological material are needed, facilitating the use of non-invasive methods and avoiding the sacrifice of the animals studied. Moreover, fresh, frozen or alcohol preserved material can be used.

The results reported in this work are a first step in the genetic characterisation of the species of the genus *Gobio*. Further steps will include the use of RAPD in studies on population genetics. RAPD markers, which mainly reveal changes in non-coding DNA regions (W i l l i a m s et al. 1990, H a y m e r & M c I n n i s 1994), have less selection constraints than allozymes. Therefore, the resolution between species and even populations may be better because of the greater polymorphism that can be detected. This information might be important in recovery programs since the identification and evaluation of bloodstocks or wild fish genotypes, the spatial genetic differentiation of populations, and the maintenance of genetic diversity over time can all be checked. Certainly, knowledge of genetic structure and of the relationships among populations is essential for understanding life history patterns, and hence is essential in the development of appropriate and efficient conservation strategies.

A c k n o w l e d g e m e n t s

This study was funded by the Grant Agency of the Academy of Sciences of the Czech Republic, grants no. A6045005 and no. A6093105.

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